EXHIBIT 6

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Adult Rat and Human Bone Marrow Stromal Cells Differentiate Into Neurons

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Bone marrow stromal cells exhibit multiple traits of a stem cell population. They can be greatly expanded in vitro and induced to differentiate into multiple mesenchymal cell types. However, differentiation to non-mesenchymal fates has not been demonstrated. Here, adult rat stromal cells were expanded as undifferentiated cells in culture for more than 20 passages, indicating their proliferative capacity. A simple treatment protocol induced the stromal cells to exhibit a neuronal phenotype, expressing neuron-specific enolase, NeuN, neurofilament-M, and tau. With an optimal differentiation protocol, almost 80% of the cells expressed NSE and NF-M. The refractile cell bodies extended long processes terminating in typical growth cones and filopodia. The differentiating cells expressed nestin, characteristic of neuronal precursor stem cells, at 5 hr, but the trait was undetectable at 6 days. In contrast, expression of trkA, the nerve growth factor receptor, persisted from 5 hr through 6 days. Clonal cell lines, established from single cells, proliferated, yielding both undifferentiated and neuronal cells. Human marrow stromal cells subjected to this protocol also differentiated into neurons. Consequently, adult marrow stromal cells can be induced to overcome their mesenchymal commitment and may constitute an abundant and accessible cellular reservoir for the treatment of a variety of neurologic diseases. J. Neurosci. Res. 61:364–370, 2000.

Key words: mesenchymal stem cells; neuronal differentiation; neurological diseases

Pluripotent stem cells have been detected in multiple tissues in the adult, participating in normal replacement and repair, while undergoing self-renewal (Hay, 1966; Kuznetsov et al., 1997; Owens and Friedenstein, 1988; Caplan, 1991; Pereira et al., 1995; Prockop, 1997; Ferrari et al., 1998; Majumdar et al., 1998; Pittenger et al., 1999; McKay, 1999; Lemiscka, 1999). A subclass of bone marrow stem cells is one prototype, capable of differentiating into osteogenic, chondrogenic, adipogenic, and other mesenchymal lineages in vitro (Kuznetsov et al., 1997; Caplan, 1991; Pereira et al., 1995; Prockop, 1997; Ferrari et al., 1998; Majumdar et al., 1998; Pittenger et al., 1999). They have been termed *marrow stromal cells* (MSCs) and recently have been used clinically to treat osteogenesis imperfecta (Horwitz et al., 1999).

The recent discovery of stem cell populations in the central nervous system (CNS) has generated intense interest, since the brain has long been regarded as incapable of regeneration (Reynolds and Weiss, 1992; Richards et al., 1992). Neural stem cells (NSCs) are capable of undergoing expansion and differentiating into neurons, astrocytes, and oligodendrocytes in vitro (Reynolds and Weiss, 1992; Vescovi et al., 1993; Gage et al., 1995a; Johansson et al., 1999). NSCs back-transplanted into the adult rodent brain survive and differentiate into neurons and glia, raising the possibility of therapeutic potential (Renfranz et al., 1991; Gage et al., 1995b; Lundberg et al., 1996, 1997; Svendsen et al., 1997; Flax et al., 1998). However, the inaccessibility of NSC sources deep in the brain severely limits clinical utility. The recent report demonstrating that NSCs can generate hematopoietic cells in vivo suggests that stem cell populations may be less restricted than was previously thought (Bjornson et al., 1999). Evidence that MSCs injected into the lateral ventricles of neonatal mice can differentiate to astrocytes and neurofilament-containing cells lends support to this contention (Kopen et al., 1999). We now report that rodent and human cultured MSCs can be induced to differentiate exclusively into neurons, potentially enhancing their usefulness in the treatment of neurological disease.

MATERIALS AND METHODS

Cell Culture

rMSCs were originally cultured in α-MEM supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 25 ng/ml amphotericin B. For each passage the cells were plated at about 8,000 cells/cm^2 and grown to confluency. At passage 6 the cells were transferred to DMEM/20% FBS without additional supplementation and were maintained beyond passage 20. The rat MSCs were obtained with a protocol and procedures approved by the Institutional IACUC. The human samples were obtained from volunteers with informed consent and according to a protocol approved by the Institutional Review Board.

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Neuronal Induction

Subconfluent cultures of rat and human MSCs were maintained in DMEM/20% FBS. Twenty-four hours prior to neuronal induction, media were replaced with preinduction media consisting of DMEM/20% FBS/1 mM β-mercaptoethanol (BME). To initiate neuronal differentiation, the preinduction media were removed, and the cells were washed with PBS and transferred to neuronal induction media composed of DMEM/1–10 mM BME. In later experiments DMEM/2% dimethylsulfoxide (DMSO)/200 μM butylated hydroxyanisole (BHA) was utilized as the neuronal induction media. Cells were fixed for immunocytochemistry at times ranging from 30 min to 6 days postinduction.

Western Blot

Thirty micrograms of protein extract from untreated (U) and BME-induced (I) rMSC cultures were separated on a 4–20% gradient acrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The blot was probed for tubulin expression (monoclonal; Sigma, St. Louis, MO), stripped, and probed for neuron-specific enolase (NSE) expression (polyclonal; ICN, Irvine, CA). Secondary antibodies were HRP conjugated and developed with ECL reagents (Amersham, Arlington Heights, IL).

Immunocytochemistry

Cultured rMSCs were fixed with 4% paraformaldehyde, incubated with primary antibody overnight at 4°C, incubated with secondary antibody for 1 hr, followed by exposure to avidin-biotin complex for 1 hr (25°C). DAB served as chromagen.

Quantitation of Neuronal Differentiation

Cultured rMSCs were pretreated overnight in DMEM/20% FBS/10 ng/ml bFGF. Neuronal differentiation was induced with DMEM/2% DMSO/200 μM BHA, and cells were fixed at 5 hr postinduction. Three independent experiments were performed in duplicate. Cells were stained for NSE or neurofilament-M (NF-M) expression. For quantitation, a Fugix digital camera was used to capture 10 non-overlapping low power images (×100) of each sample. Cells exhibiting retracted cell bodies and strong NSE or NF-M staining were counted and compared to total cell counts, and the mean and standard deviation were computed.

Maintenance of Long-Term Neuronal Cultures

To facilitate long-term survival of rMSC-derived neurons several additional components were added to the neuronal induction media. Preinduction was carried out as described above under Quantitation of Neuronal Differentiation. Long-term neuronal induction media consisted of DMEM/2% DMSO/200 μM BHA/25 mM KCl/2 mM valproic acid/10 μM forskolin/1 μM hydrocortisone/5 μg/ml insulin.

RESULTS

Stromal Cell Characterization

rMSCs were isolated from the femurs of adult rats and propagated in vitro (Azizi et al., 1998). Fluorescent cell sorting at passage 1 demonstrated that the cells were negative for CD11b (Fig. 1.1) and CD45 (Fig. 1.2), cell surface markers associated with lymphohematopoietic cells. Therefore, there was no evidence of hematopoietic precursors in the cultures. In contrast, the rMSCs did express CD90 (Fig. 1.3), consistent with their undifferentiated state. At low plating densities, rMSCs grew as a monolayer of large, flat cells. As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology. At the outset of the neuronal differentiation studies (see below), untreated rMSCs were further characterized by staining for the cell surface markers CD44 and CD71. Cells were positive for CD44 and CD71 expression, consistent with previous reports (Bruder et al., 1998; Pittenger et al., 1999; data not shown).

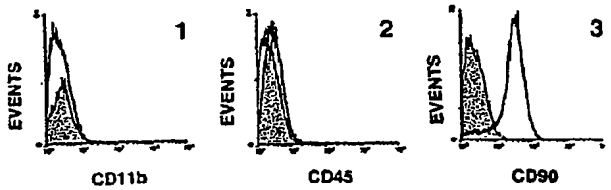

Fig. 1. Characterization of undifferentiated rMSCs. Fluorescent cell sorting of passage 1 rMSCs employing monoclonal antibodies directed against cell surface markers (open peaks). Secondary antibody is FITC-conjugated anti-mouse antibody. An isotype control is included in each experiment to identify background fluorescence (shaded peaks). Number of cells analyzed (events) is plotted on the y-axis; intensity of staining is plotted on the x-axis. 1: CD11/integrin α_m /Mac-1 α chain (Pharmingen). Distribution of cells stained with antibody to CD11b (open) does not differ from that of isotype control (shaded), indicating the rMSC cultures do not contain significant numbers of contaminating CD11b-expressing cells. 2: CD45/leukocyte common antigen (Pharmingen). Intensity of staining does not differ between CD45 antibody (open) and control (shaded) profiles, indicating that cultured rMSCs are not contaminated by CD45-expressing cells. 3: CD90/Thy-1/CD90.1/Thy1.1 (Pharmingen). Fluorescence intensity is greater (shifted to the right) when rMSCs are incubated with CD90 antibody (open) compared to control antibody (shaded). The vast majority of cells in the rMSC cultures express CD90, consistently with their undifferentiated state.

Neuronal Differentiation

To induce the neuronal phenotype, rMSCs were maintained in subconfluent cultures in serum-containing medium supplemented with 1 mM β-mercaptoethanol (BME) for 24 hr. To effect neuronal differentiation, the cells were transferred to serum-free medium containing 1–10 mM BME (SFM/BME). Within 60 min of exposure to SFM/BME, changes in morphology of some of the rMSCs were apparent (arrowhead in Fig. 2). Responsive cells progressively assumed neuronal morphological characteristics over the first 3 hr. Initially, cytoplasm in the flat rMSCs retracted towards the nucleus, forming a contracted multipolar, cell body, leaving membranous, process-like extensions peripherally (0–90 min). Cells exhibited increased expression of the neuronal marker NSE within 30 min of treatment (data not shown). Over the subsequent 2 hr, cell bodies became increasingly spherical

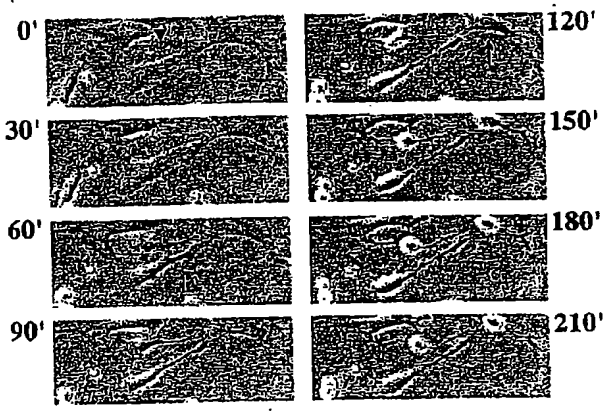

Fig. 2. Neuronal differentiation of rMSCs. The neuronal differentiation protocol was initiated at 0' and followed for 210'. A flat rMSC at 0' is identified (▼) prior to differentiation. Retraction of cell body and process elaboration is evident with increasing time. The arrow at 120' indicates a second differentiating cell. ×200.

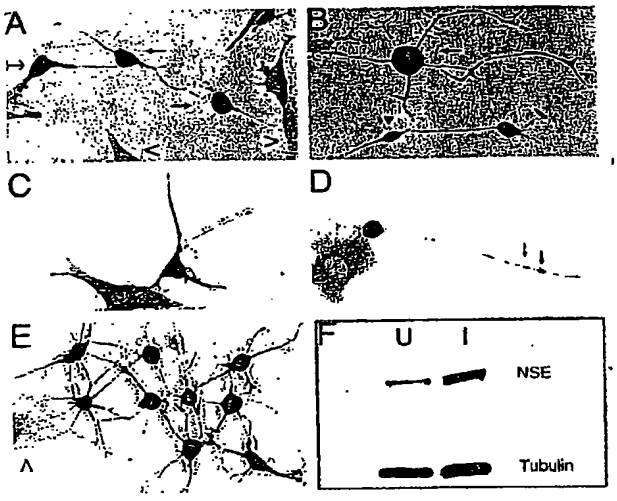

Fig. 3. NSE expression in differentiating neurons (polyclonal; Polysciences Inc.). A: Undifferentiated rMSCs (>) retain flattened morphology and stain only slightly for NSE expression. rMSC-derived neurons (thick arrows) stain dark brown for NSE expression and display condensed cell bodies and highly branched processes. Transitional cells (thin arrow at left) exhibit intermediate neuronal morphologies, with partially retracted cell bodies and light brown NSE staining. B: Morphologies of rMSC-derived neurons include simple bipolar cells (arrowhead) and complex multipolar cells with highly branched processes (arrow). Intense NSE staining is evident in both neuronal cell types. C: NSE-positive neurons displaying pyramidal morphologies are sometimes generated. Contact with a transitional cell (light brown) is maintained via a single unbranched process. D: NSE-positive neuron elaborating a long process with evident varicosities (arrows). The neuronal cell body is in intimate contact with a transitional cell. E: Clusters of rMSC-derived neurons of varying morphologies form complex networks. An undifferentiated rMSC (>) is included within this meshwork of processes. ×320. F: Western blot analysis confirms expression of low levels of NSE in uninduced rMSCs (U). A significant increase in NSE expression is evident at 5 hr after BME treatment (I). Comparable levels of tubulin are detected in each lane, indicating equal loading of samples.

and refractile, exhibiting a typical neuronal perikaryal appearance. Processes continued to elaborate, displaying primary and secondary branches, growth cone-like terminal expansions and putative filopodial extensions (see Fig. 2, 180' and 210' for examples). A second rMSC (Fig. 2, arrow) demonstrates a delayed but equally dramatic response to SFM/BME treatment.

To characterize neuronal differentiation further we fixed BME-treated cultures after 5 hr and stained them for the neuronal marker NSE. Unresponsive, flat rMSCs expressed very low, but detectable, levels of NSE protein, consistent with previous detection of minute amounts of protein and/or message in cells of bone marrow origin (van Obberghen et al., 1988; Reid et al., 1991; Pechumer et al., 1993). Progressive transition of rMSCs to a neuronal phenotype coincided with increased expression of NSE (Fig. 3A). Cells that exhibited contracted cell bodies and processes stained dark brown for NSE expression (Fig. 3A, large arrows), whereas flat, unresponsive rMSCs (Fig. 3A, >) displayed minimal NSE staining. Cells at intermediate stages in the differentiation sequence (Fig. 3A, small arrow at left) exhibited transitional morphologies and light brown staining, indicating synchrony of morphologic and molecular differentiation. rMSC-derived neurons displayed distinct neuronal morphologies (Fig. 3B), ranging from simple bipolar (Fig. 3B, arrowhead) to large, extensively branched multipolar cells (Fig. 3B, arrow). Rare NSE-positive neurons exhibited pyramidal cell morphologies (Fig. 3C), whereas neurons elaborating long processes with evident varicosities (Fig. 3D, arrows) were more common. Clusters of differentiated cells exhibited intense NSE positivity, and processes formed extensive networks (Fig. 3E). Even within these clusters, typical, flat rMSCs (Fig. 3E, >) were only lightly stained, consistent with their undifferentiated state. Western blot analysis confirmed the expression of low levels of NSE protein in uninduced rMSCs. Induction of the neuronal phenotype resulted in a dramatic increase in NSE expression, consistent with the immunocytochemical data (Fig. 3F).

Although the precise mechanisms by which BME induces neuronal differentiation are unclear, its antioxidant properties, which enhance neuronal survival in vitro (Ishii et al., 1993), may be partially responsible for the present neuronal induction. To begin examining this hypothesis, we treated rMSCs with DMSO, BHA, or butylated hydroxytoluene (BHT) alone and in combination. Each treatment elicited neuronal morphologies with a time course similar to that of BME. Preliminary data suggested that treatment with 2% DMSO and 200 μM BHA (DMSO/BHA) was most effective (data not shown). To characterize neuronal identity further, MSCs treated

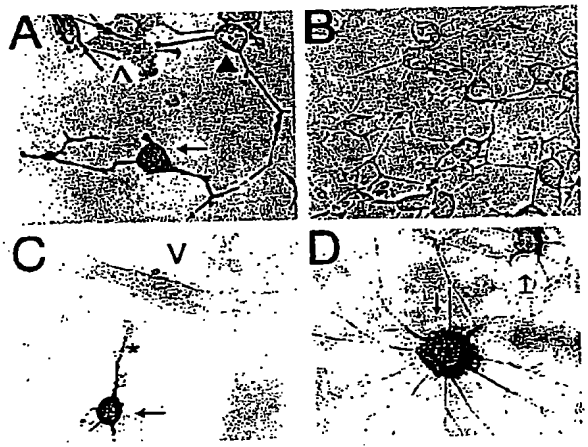

Fig. 4. Expression of NF-M, tau, and NeuN by differentiating cells. A: rMSC-derived neurons stained for expression of NF-M (polyclonal; Chemicon). Most cells that exhibit neuronal morphologies express NF-M in both cell bodies and processes (arrow). In a subset of rMSC-derived neurons (arrowhead), the cell body is devoid of staining, and NF-M staining is localized exclusively to the processes. Flat, undifferentiated rMSCs (>) do not stain for NF-M expression. B: Preadsorption of NF-M antibody with 20 μg of purified NF-M protein overnight at 4°C eliminated staining of rMSC-derived neurons, indicating specificity of the NF-M staining. C: rMSC-derived neurons stained for expression of tau (polyclonal; Sigma). Cells displaying neuronal morphologies (arrows) stain dark brown for tau expression within the cell body and extending into the processes (asterisk). Flat, undifferentiated rMSCs (>) do not express tau and are unstained. D: NeuN can be detected in the nucleus and surrounding cytoplasm of a rMSC-derived neuron (arrow pointing downward). Staining does not extend into the processes of positive cells. Some cells exhibiting neuronal morphologies (arrow pointing upward) do not express NeuN. ×320.

with DMSO/BHA were stained for NF-M, a neuron-specific intermediate filament that helps to initiate neurite elongation (Carden et al., 1987). We previously found that BME treatment of MSCs caused increased expression of NF-M in cells exhibiting neuronal morphologies (data not shown). Most cells displaying rounded cell bodies with processes (Fig. 4A, arrow) after DMSO/BHA exposure expressed high levels of NF-M, whereas flat, undifferentiated cells (Fig. 4A, >) did not. A subset of rMSC-derived neurons partitioned NF-M antigen exclusively to the processes (Fig. 4A, arrowhead), indicative of a maturing neuronal phenotype (Benson et al., 1996). Preadsorption of NF-M antibody with purified NF-M protein abolished staining (Fig. 4B), establishing specificity.

We next examined DMSO/BHA-treated cultures for the presence of tau, a neuron-specific microtubule-associated protein expressed by differentiating neurons (Kosik and Finch, 1987). Cells exhibiting a neuronal morphology (Fig. 4C, arrow) expressed tau protein in the cell body as well as in the processes (Fig. 4C, asterisk), whereas undifferentiated flat cells were tau-negative (Fig. 4C, >). In contrast to the expression of neuronal gene products, cells did not express the classical glial astrocyte marker glial fibrillary acidic protein (GFAP; data not shown). We tentatively conclude that our protocol(s) induce neuronal, but not astrocytic differentiation.

To investigate neuronal characteristics further, we stained differentiated cultures for NeuN, a neuron-specific marker expressed in postmitotic cells (Sarnat et al., 1998). A subset of cells exhibiting rounded cell bodies and processes (Fig. 4D, arrow pointing downward) stained for NeuN expression, whereas neighboring cells exhibiting distinct neuronal morphologies were NeuN-negative (Fig. 4D, arrow pointing upward). This pattern contrasts with that established for NSE staining, in which every cell exhibiting a neuronal morphology demonstrated increased NSE expression. This observation suggests that a subset of NSE-positive cells are postmitotic neurons.

Quantitation of Neuronal Differentiation

With the foregoing induction protocol, a variable number of cells underwent neuronal differentiation, though the response generally exceeded 50%. To optimize differentiation, we modified the preinduction protocol. Addition of bFGF (10 ng/ml) and elimination of BME from the preinduction media increased the proportion of cells displaying neuronal characters, and the response was more consistent within and between experiments. To quantitate this response, rMSCs were treated with the modified preinduction protocol and induced to differentiate with DMSO/BHA as described above. Cells were fixed after 5 hr, stained for the neuronal markers NSE and NF-M, and the percentage of neuronal cells was determined. The majority of rMSCs treated in this manner exhibited neuronal morphologies and stained positive for NSE (78.2% ± 2.3%) and NF-M (79.2% ± 2.5%) expression.

Long-Term Differentiation

A striking feature of rMSC neuronal differentiation is the rapidity of the response. As a result, our analysis has focused on the changes that occur within the first 5 hr of differentiation. However, long-term differentiation of these cells will be important to our understanding of this process. To address this issue, we monitored expression of the nestin gene product in rMSC-derived neurons at 5 hr, 1 day, and 6 days post-differentiation (Fig. 5). Nestin, an intermediate filament protein, is expressed in neuroepithelial neuronal precursor stem cells, and its expression decreases with neuronal maturation (Lendahl et al., 1990). A subset of rMSC-derived neurons expressed high levels of nestin protein at 5 hr, and the proportion of nestin-positive cells decreased with time. By 6 days post-induction there was no detectable nestin expression in any rMSC-derived neurons, consistent with ongoing maturation with time.

We compared the time course of expression of the transitional trait, nestin, to that of a mature phenotypic character, trkA, the high-affinity, biologically active nerve growth factor receptor. TrkA was detectable at 5 hr, the earliest time examined and, in contrast to nestin, persisted unchanged through 6 days.

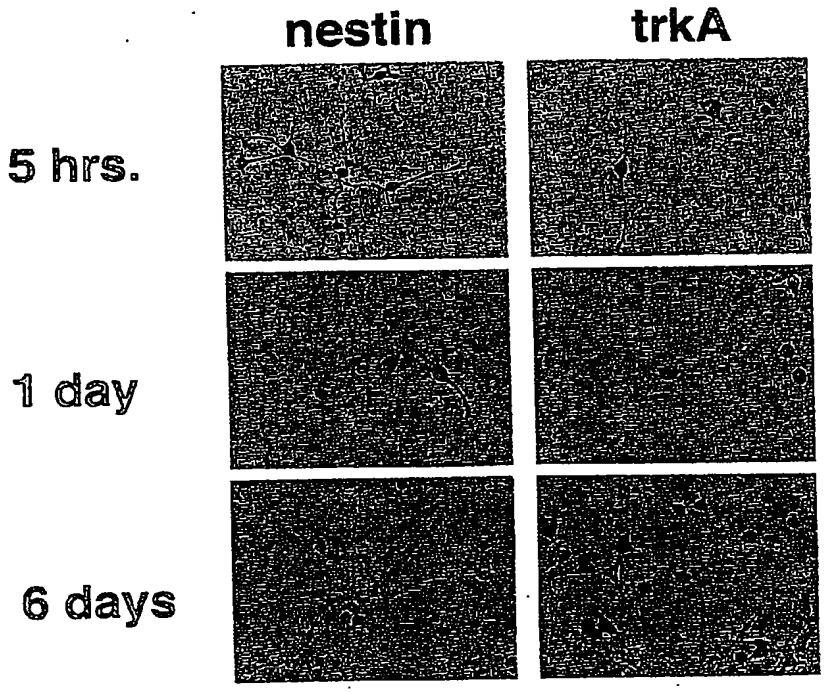

Fig. 5. Expression of nestin and trkA in differentiating rMSC-derived neurons. rMSC-derived neurons were fixed at 5 hr, 1 day, or 6 days postinduction and stained for nestin or trkA expression. Cells display decreasing nestin expression as they mature, whereas trkA levels remain unchanged. ×320.

Clonal Analysis

To determine whether individual rMSCs exhibit stem cell characteristics of self-renewal and pluripotentiality, individual clones were analyzed. To establish clones, rMSCs were plated at approximately 10 cells/cm^2, grown to 50–150 cells per colony, isolated with cloning cylinders, and transferred to separate wells and finally to individual flasks. Single cells replicated as typical rMSCs and differentiated into NSE-positive neurons after BME treatment. Analysis of four distinct clonal lines is shown in Figure 6. Each individual clone generated refractile, process-bearing, NSE-positive cells following BME treatment. Undifferentiated rMSCs (Fig. 6, >) and transitional cells (Fig. 6, arrow) were evident in each clonal line. Therefore, clones derived from single cells give rise to both rMSCs and neurons, indicating stem cell characteristics.

Human Stromal Cells Differentiate Into Neurons

We examined whether the neuronal potential of MSCs was unique to rodents or whether human MSCs (hMSCs) shared this capacity. hMSCs were isolated from a healthy adult donor and grown in vitro (Azizi et al., 1998). hMSCs resembled their rodent counterparts, growing as large, flat cells in the undifferentiated state. Cells from passage 2 were subjected to the neuronal differentiation protocol and stained for NSE or NF-M expression. After BME treatment, hMSCs exhibited neuronal characteristics and increased NSE expression in a time frame similar to that observed for rMSCs. Contracted cell bodies elaborated processes and stained intensely for NSE expression within 3 hr (Fig. 7A,B). Transitional cells were also evident (Fig. 7A,B, arrows pointing leftward). Many processes elaborated by hMSC-derived neurons exhibited terminal bulbs (Fig. 7B, arrow pointing downward), which may represent growth cones. Growth cone morphologies with filopodial extensions (Fig. 7C, double-headed arrow) were clearly evident on the processes elaborated by paired neurons in Figure 7C. These cells also expressed NF-M, consistent with their neuronal differentiation (Fig. 7D).

DISCUSSION

Our observations indicate that rat and human MSCs retain the capacity to differentiate into nonmesenchymal derivatives, specifically neurons, suggesting that intrinsic genomic mechanisms of commitment, lineage restriction, and cell fate are mutable. Environmental signals apparently can elicit the expression of pluripotentiality that extends well beyond the accepted fate restrictions of cells originating in classical embryonic germ layers. These adult cells are

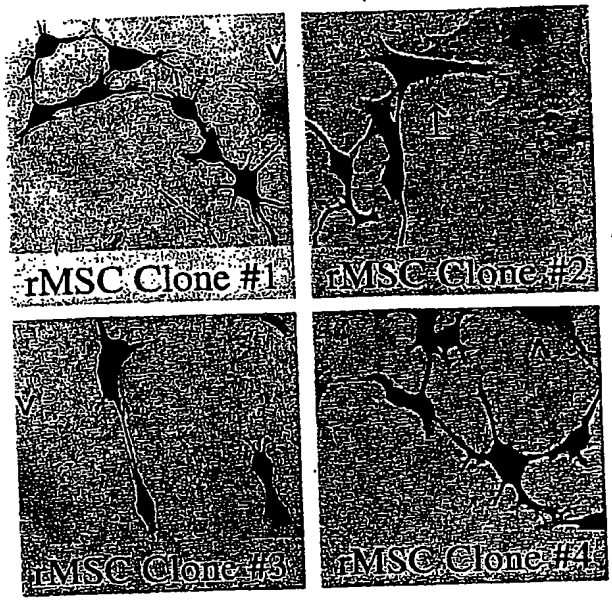

Fig. 6. Differentiation of clonal rMSC lines. NSE staining of individual rMSC clones (1–4) subjected to the differentiation protocol. NSE-positive cells (dark brown) are derived from each clonal line. Undifferentiated rMSCs (>) and/or transitional cells (arrow) are evident in each panel. ×320.

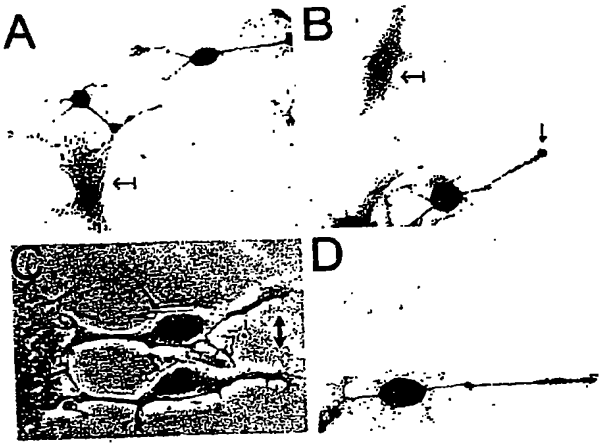

Fig. 7. Differentiation of human MSCs. A: Human MSCs differentiate into neurons and express high levels of NSE (dark brown). A more lightly stained transitional cell (arrow) is visible at lower left. B: NSE-positive hMSC-derived neuron elaborates a process with terminal bulb morphology. C: Phase-contrast image of paired NSE-positive neurons demonstrates growth cone morphologies with filopodial extensions (double-headed arrow). Image enlarged 50% to show detail. D: hMSC-derived neurons stain positive for NF-M. ×320.

both self-renewing and multipotential (Kuznetsov et al., 1977; Caplan, 1991; Pereira et al., 1995; Prockop, 1997; Ferrari et al., 1998; Majumdar et al., 1998; Pittenger et al., 1999), thereby fulfilling many of the criteria of a stem cell population.

To our knowledge, this is the first report that peripheral mesenchymal cells can differentiate into neurons in vitro. MSCs may be useful in the treatment of a wide variety of neurologic diseases, offering significant advantages over other "stem" cells. The marrow cells are readily accessible, overcoming the risks of obtaining neural stem cells from the brain, and provide a renewable population. Autologous transplantation overcomes the ethical and immunologic concerns associated with the use of fetal tissue. Moreover, MSCs grow rapidly in culture, precluding the need for immortalization, and differentiate into neurons exclusively with use of a simple protocol.

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